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Neurotoxic effects of paraoxon and isofenphos on the ventral nerve cord of the American cockroach, Periplaneta americana

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NEUROTOXIC EFFECTS OF PARAOXON AND ISOFENPHOS ON THE VENTRAL NERVE CORD OF THE AMERICAN COCKROACH, PERIPLANETA AMERICANA

Iowa State University PH.D. 1986

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Neurotoxic effects of paraoxon and isofenphos on the ventral nerve cord of the American cockroach, Periplaneta americana

by

Thomas Jon Heppner

A Dissertation Submitted to the

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For the Graduate College

Iowa State University
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1986

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GENERAL INTRODUCTION

Most major classes of insecticides in use today are neurotoxicants. The organophosphates (OPs), one of the largest insecticide classes, are widely used in agriculture. The major mechanism of OP toxicity has been attributed to inhibition of the enzyme acetylcholinesterase. Studies with vertebrate and invertebrate systems have shown strong correlations between cholinesterase inhibition, behavioral symptomology and electrophysiological events.

Product development and usage of one group of OPs, the phosphoramidates, have increased in the last twenty years. Although these compounds have good insecticidal properties, the neurotoxic effects and mechanisms of action of most phosphoramidates, including a relatively new one, isofenphos (tradenames Amaze^{R} and Oftanol^{R}), have not been extensively studied. A two-part study was undertaken to help elucidate the neurotoxic actions of isofenphos.

Part I. Because the mode of action of most OPs involves cholinesterase inhibition, isofenphos and three of its metabolites were examined for their ability to inhibit cholinesterase and induce abnormal electrical activity in the ventral nerve cord (VNC) of the American cockroach. Cholinesterase activity was correlated with the induction of electrical activity in the VNC. Both in vitro and in vivo cholinesterase assays were used, the latter method allowing assessment of cholinesterase activity in the intact VNC.

Part II. The electrophysiological effects of the OP, paraoxon were

examined on the cholinergic cercal afferent-to-giant interneuron synapse in the central nervous system of the American cockroach. This is the first study in which intracellular microelectrodes have been used to assess the action of OPs on identified insect neurons.

Explanation of Dissertation Format

The dissertation is written in the alternate format. Each major division (Parts I and II) is a complete manuscript modified to conform with the specifications of the Iowa State University Thesis Office. Each part has its own introduction, materials and methods, results, discussion, and references. Following these two parts is a general summary of the entire dissertation.

PART I. NEUROTOXIC ACTIONS OF ISOFENPHOS AND THREE ISOFENPHOS METABOLITES IN THE AMERICAN COCKROACH, PERIPLANETA AMERICANA

INTRODUCTION

The primary mode of action of organophosphorus insecticides (OPs) in both insects and mammals is the inhibition of acetylcholinesterase. The binding of the OP to cholinesterase causes the accumulation of the neurotransmitter acetylcholine in the synaptic cleft (Corbett et al., 1984). Because synaptic transmission between the cercal afferent fibers and at least some of the ventral giant interneurons (GIs) in the American cockroach Periplaneta americana is cholinergic (for review see Sattelle, 1980; Sattelle and Harrow, 1983), the spontaneous bursting and prolonged after-discharge of GIs and other units observed after exposure to OPs have been attributed to cholinesterase inhibition (Colhoun, 1960; Narahashi and Yamasaki, 1960; Yamasaki and Narahashi, 1960; Burt et al., 1966).

Although the neurotoxic effect of some OPs is exerted before any metabolic alterations in chemical structure, others must first be activated to a more toxic form. For example, the thionophosphate, parathion, is a poor inhibitor of acetylcholinesterase in vitro (Diggle and Gage, 1951a; Metcalf and March, 1953a), but a good inhibitor in vivo (Diggle and Gage, 1951b). Inhibition of acetylcholinesterase in this case is attributed to the effect of paraoxon, the oxon metabolite of parathion (Metcalf and March, 1953b). Capabilities for the conversion of parathion to paraoxon vary considerably in different insect tissues, the greatest conversion occurring in the foregut, followed by the malpighian tubules, midgut, nerve cord, hindgut, and fat body (Metcalf and March,

1953b).

The phosphoramidates are very potent insecticides (Magee, 1982), but their mode of action is still unclear. Studies of the relationships between toxicity and cholinesterase inhibition in vitro for a series of phosphoramidates (Neely and Whitney, 1968), phosphoramidothiolates (Quistad et al., 1970), and phosphoramidothioates (Sanborn and Fukuto, 1972) have shown that some of these compounds were moderate to strong inhibitors of cholinesterase. However, there was not always a strong correlation between toxicity and cholinesterase inhibition for some of these compounds.

The phosphoramidothionate, isofenphos, is a soil insecticide recently marketed under the trade names Amaze^{R} and Oftanol^{R}. Metabolic studies of isofenphos in the southern corn rootworm (Diabrotica undecimpunctata) showed that one of the major metabolic pathways of isofenphos was oxidative desulfuration to isofenphos oxon, with subsequent hydrolysis to isopropyl salicylate (Hsin and Coats, in press). Small amounts of des-N-isopropyl isofenphos (DNI) and des-N-isopropyl isofenphos oxon (DNIO) were also found, along with unidentified polar metabolites. Our preliminary studies indicated that the oxidation products induced neurophysiological activity similar to paraoxon (Heppner et al., 1985). In this study, we have examined the ability of isofenphos, isofenphos oxon, DNI, and DNIO to inhibit cholinesterase (in vivo and in vitro) and induce high-frequency bursting activity within the ventral nerve cord (VNC) of Periplaneta americana.

METHODS AND MATERIALS

Animals

Cultures of Periplaneta americana were maintained at 27°C in screened enclosures and fed Purina® dog chow and water. Only adult male cockroaches, of varying ages, were used for electrophysiology and cholinesterase experiments.

Compounds and Solvents

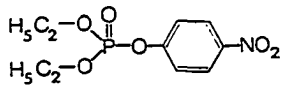
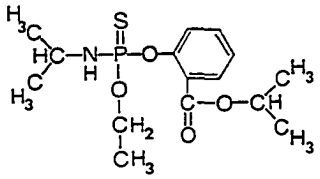
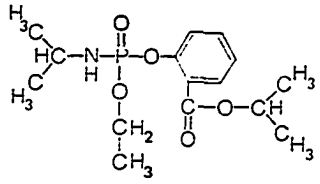
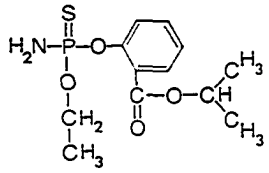
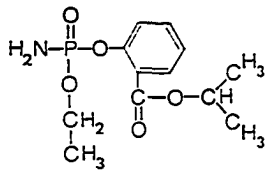
Paraoxon, isofenphos (O-ethyl O-(2-isopropoxycarbonyl)phenyl isopropylphosphoramidothioate), and three of its metabolites, DNIO, DNI, and isofenphos oxon (Figure 1) were provided by Mobay Chemical Corporation, Kansas City, MO. These compounds were stored in a freezer and before use were checked for purity by thin-layer chromatography. R_f values are shown in Figure 1. Each compound was dissolved in dimethyl sulfoxide (DMSO), then either added to buffer for in vitro cholinesterase assays or diluted in normal cockroach saline (Sattelle and Harrow, 1983) for electrophysiology experiments and in vivo cholinesterase assays. The highest concentration of DMSO used in the electrophysiology experiments was 0.5% and, in the cholinesterase assays, 0.3%. In independent experiments without the insecticide, 0.5% DMSO induced no activity in the VNC.

Electrophysiology

To study electrophysiological effects of isofenphos and its metabolites, the VNC of an adult male American cockroach was exposed by

Figure 1. Structures and R_f values for the compounds used in electrophysiology and cholinesterase assays

Paraoxon, Isofenphos, and Three Isofenphos Metabolites

Compound	Structure	R_f Hexane/Acetone (4:3)
Paraoxon		0.43
Isofenphos		0.54
Isofenphos oxon		0.31
DNI		0.45
DNIO		0.14

removal of the dorsal abdominal cuticle and gut. Tracheal connections and nerve roots attached to the VNC remained intact. Normal saline was placed in the body cavity, and the preparation was allowed to stabilize for 5-10 min. Then the normal saline in the body cavity was replaced with saline containing one of the inhibitors; only one concentration was used per preparation. During treatment, the electrical activity within the VNC was recorded with a pair of silver-wire hook electrodes placed beneath the VNC between the 5th and 6th abdominal ganglia. The relative neurotoxic potency of these compounds was determined by measuring the time from application until the onset of high-frequency spontaneous bursting in the VNC. Intracellular recordings from GIs were obtained by using conventional glass microelectrodes filled with 1.0 M LiCl and the fluorescent dye lucifer yellow. At the termination of some experiments, the dye was iontophoresed into the impaled GI, enabling identification based on dendritic morphology and cell body location (Daley et al., 1981).

Homogenate Preparation

For the determination of cholinesterase activity, the entire nerve cord, beginning at the first thoracic and including the sixth abdominal ganglion, was removed from unanesthetized roaches under 0.1 M ice-cold sodium phosphate buffer (pH 7.4). Fat and large tracheae were removed from the VNC. The nerve cord was then homogenized in cold buffer (concentration = 5 VNCs per 4 ml buffer) by using a glass tissue homogenizer and centrifuged for 5 min at 748 xg to remove large debris.

The supernatant of the homogenate was used immediately or frozen at -15°C for later use. At this temperature, cholinesterase activity was unchanged over a period of at least 2 weeks.

Cholinesterase Activity

Cholinesterase activity was determined by the colorimetric method of Ellman et al. (1961) by using acetylthiocholine iodide (ATChI) as the substrate. Temperature and pH were 30°C (Edwards, 1980) and 7.4 (Stegwee, 1951), respectively. Because ATChI undergoes spontaneous hydrolysis, substrate solutions were prepared immediately before use. To correct for nonenzymatic hydrolysis of the substrate during the assay, a reference tube containing the same substrate concentration as the sample tube was maintained as a blank. To ensure the accuracy of the assay, a control tube containing no inhibitor was also utilized for each assay. Preliminary experiments showed maximal enzyme activity at substrate concentrations between 4×10^{-3} M and 5.6×10^{-3} M, agreeing closely with the previously reported value of 5.13×10^{-3} M (Edwards, 1980). Although some investigators have found that the color reagent, dithiobisnitrobenzoic acid (DTNB), used in the Ellman cholinesterase procedure can be inhibitory when incubated with insect cholinesterase (Zahave et al., 1972; Smissaert, 1976; Manulis et al., 1981), cholinesterase activity was not inhibited at the DTNB concentration used in the assays (3.2×10^{-4} M DTNB).

To determine the cholinesterase activity *in vitro* (n = 4-6 replicates for each concentration), 3.0 ml of phosphate buffer (pH 7.4),

0.1 ml DTNB, 20 ul of VNC homogenate, and 10 ul DMSO-inhibitor solution (final DMSO concentration = 0.3%) were incubated in a 30°C water bath for 10 min. The reaction was started by the addition of 32 ul of 0.5 M substrate. Absorbance values from the reaction tubes were obtained by using a Beckman model DB spectrophotometer. Measurements made at 5-min intervals indicated that the reaction rate was linear for at least the first 25 min. The rate of ATChI hydrolysis was calculated (Ellman et al., 1961) and expressed as uM (mg protein)$^{-1}$ h^{-1}. Protein determination of the homogenate was by the Lowry method (Lowry et al., 1951).

To determine the VNC cholinesterase activity *in vivo* (n = 4 replicates for each concentration), a saline solution containing inhibitor was added to the dissected cockroach preparation, as described for electrophysiology experiments. After 15 min incubation at 23.5°C, the VNC was removed under ice-cold sodium phosphate buffer and homogenized. Cholinesterase activity was determined as described for the *in vitro* assay.

To determine cholinesterase inhibition by the five inhibitors in the absence of mixed-function oxidase (MFO) systems, dissected preparations were pretreated for 10 min with 100 ppm B-diethylaminoethyl diphenylpropylacetate (SKF 525A), an inhibitor of MFO enzymes (n = 4 replicates for each inhibitor concentration). SKF 525A was obtained from Smith Kline and French Laboratories, Philadelphia, Pennsylvania. After a saline rinse, the cholinesterase inhibitor was placed on the preparation for 15 min, followed by VNC removal and homogenization, as described for the *in vitro* cholinesterase assay.

Because different temperatures were used for *in vitro* and *in vivo* incubations, comparisons were made between the percentages of cholinesterase inhibition at 23.5°C, and 30°C. Although the reaction rate was slower at 23.5°C, the percentage of cholinesterase inhibition at any given concentration of inhibitor was virtually identical when expressed as a percentage of control rate at either of the two temperatures.

RESULTS

Electrophysiology Assay

The application of each compound to the dissected preparation induced high-frequency bursting activity in the VNC. The duration of individual bursts usually ranged from 0.5 to 2.0 s. Such activity was derived from numerous units, as indicated by consistent differences in amplitudes of the extracellularly recorded spikes (Figure 2). During bursting, the spike frequency of individual units increased rapidly, approaching maximal frequencies of nearly 300 Hz. In many preparations, bursting occurred in a cyclical fashion, with episodes of bursting followed by quiescent periods of variable duration. Occasionally, bursts appeared to involve an orderly recruitment of units that followed the same sequence in successive bursts. In other cases, bursts in different units appeared independent of one another (Figure 3).

The exceptionally large amplitudes (up to 0.65 mV) of the extracellularly recorded spikes suggested that some of the units were GIs. This idea was tested directly by obtaining simultaneous extracellular and intracellular recordings from ventral GIs during paraoxon treatment. Figure 3 shows an example of one of these recordings and demonstrates a clear-cut temporal correlation between the intracellularly recorded GI spikes and one of the extracellularly recorded units in a burst. Such correlations were found for both GI1 and GI2, indicating that at least some of the induced bursting activity involved ventral GIs.

Figure 2. Electrical activity in the VNC before and after treatment with DNIO

A) Background activity immediately before treatment. B) Activity 9.5 min after treatment with 10^{-5} M DNIO. High-frequency bursting was induced in at least two different units (arrows)

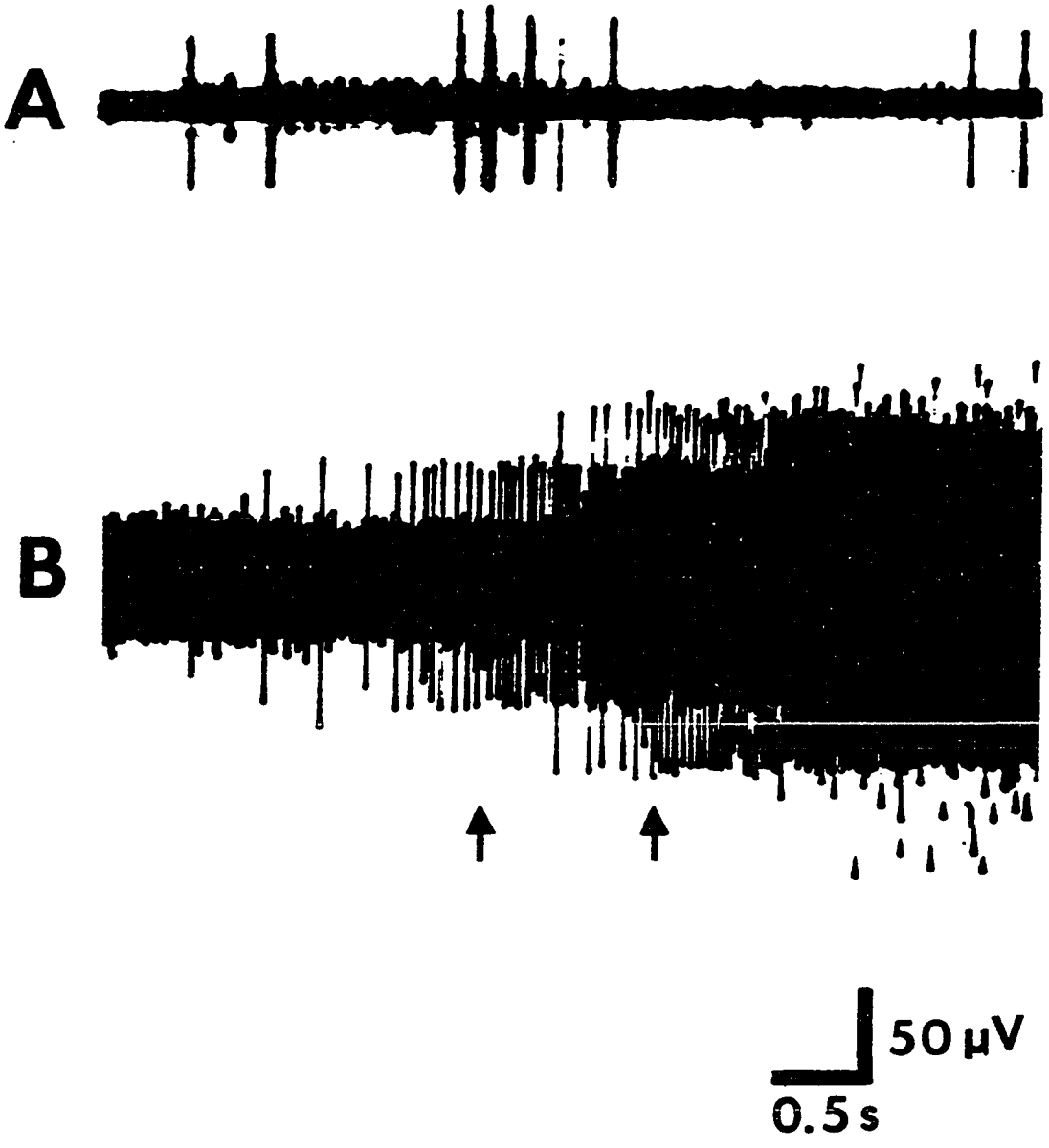
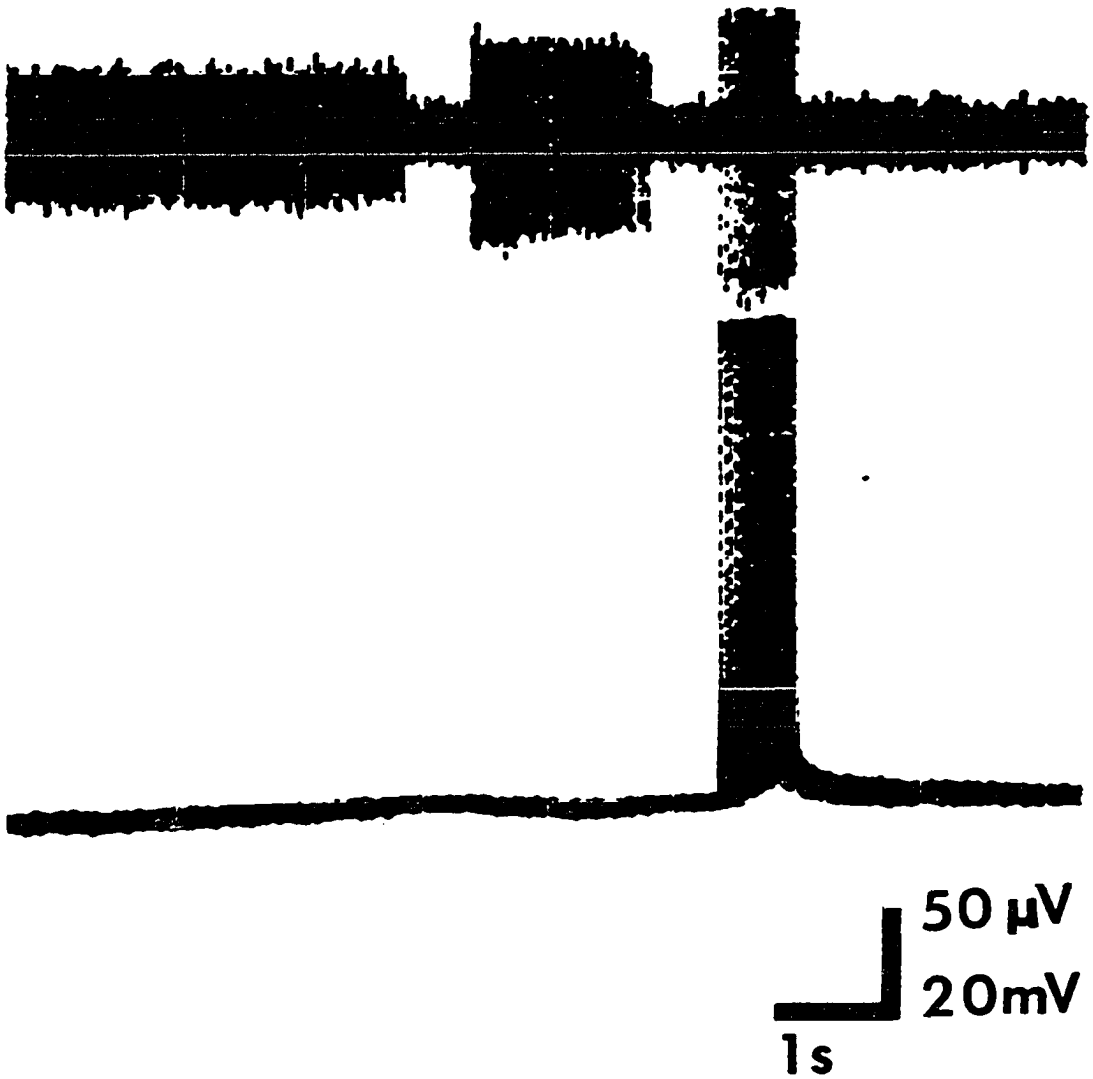

A
B
50 µV
0.5 s

Figure 3. Simultaneous recordings of extracellular activity in the abdominal connectives (top trace) and intracellular activity in a ventral GI (bottom trace), 4 min after the application of 10^{-5} M paraoxon

Bursting was evident in three different units. The largest spike amplitude corresponded to the impaled GI


50 µV
20mV
1s

Although the duration and spike frequency were often variable and difficult to quantify, the time from inhibitor application until the onset of bursting was easily measured; it varied according to the compound in a dose-dependent manner. To assess electrophysiological potency, these onset times were measured and plotted against each of four concentrations, yielding the dose-response relationship shown in Figure 4. The estimated onset times at 3.16×10^{-5} M (pI = 4.5) were 2.3 ($\pm$ 0.53 SD; n = 6), 10.8 ($\pm$ 1.60 SD; n = 6), 32.5 ($\pm$ 8.19 SD; n = 6), 40 ($\pm$ 11.40 SD; n = 6), and 85 ($\pm$ 17.47 SD; n = 3) min for paraoxon, DNIO, isofenphos oxon, DNI, and isofenphos, respectively. Dose-response data for isofenphos were incomplete because it was insoluble at a concentration of 10^{-4} M and did not induce bursting within 2 h at a concentration of 10^{-6} M (n = 3).

In Vitro Cholinesterase Assay

Comparison of the in vitro cholinesterase inhibition by the five compounds showed that only paraoxon and DNIO inhibited in a dose-dependent manner (Figure 5). The I_{50} values for these compounds were 3.1×10^{-7} M (95% CI = 2.24×10^{-7} - 3.98×10^{-7}) and 3.1×10^{-6} M (95% CI = 1.66×10^{-6} - 5.62×10^{-6}), respectively. Isofenphos, isofenphos oxon, and DNI showed some inhibition of cholinesterase, but no values were determined because there were no clear-cut dose-reponse relationships, and inhibition never exceeded 24% of control activity. To test the possibility that these results were related to solubility problems, identical concentrations of isofenphos, isofenphos oxon, and

Figure 4. Time to the onset of bursting versus concentration of the inhibitor

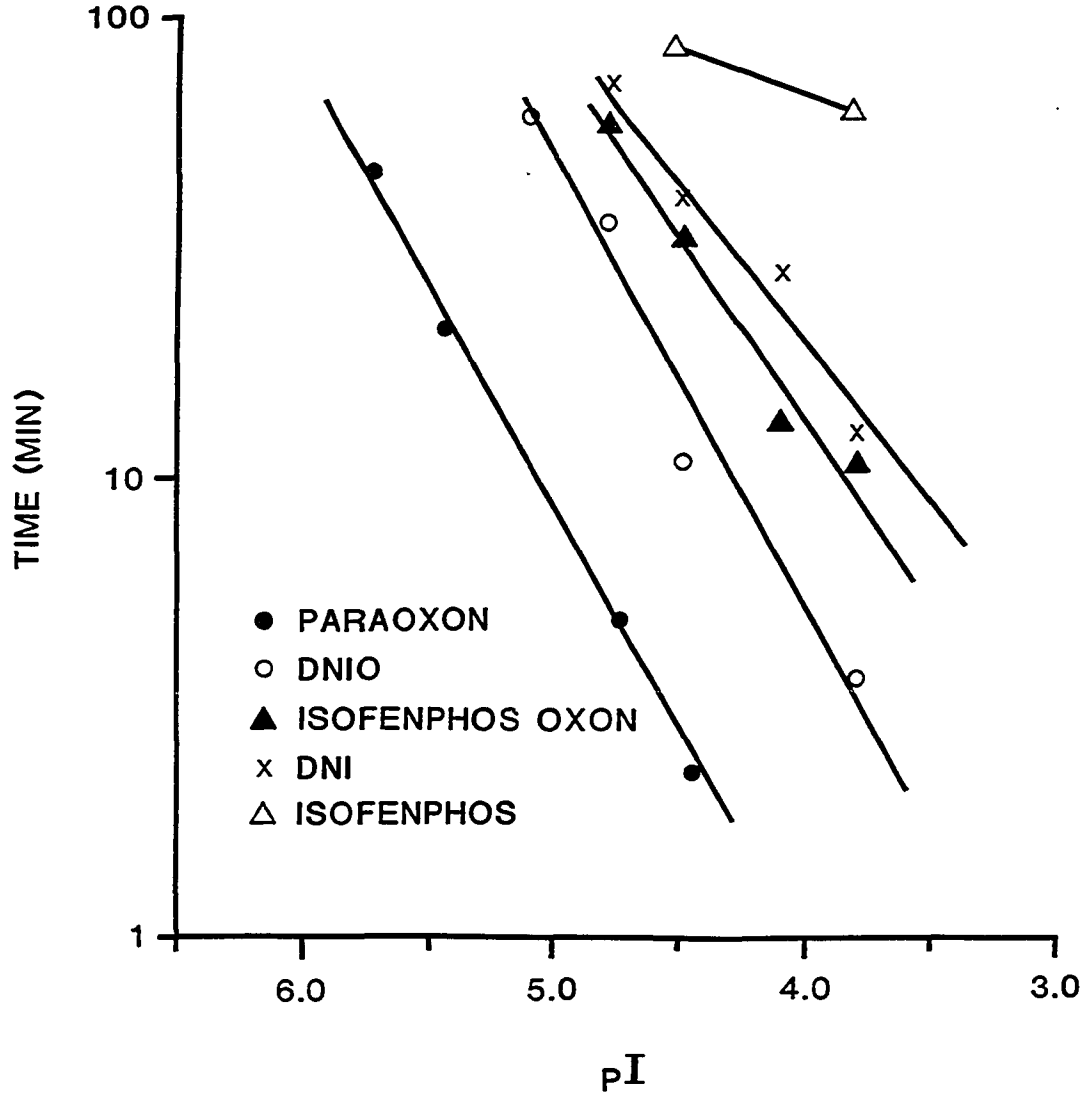
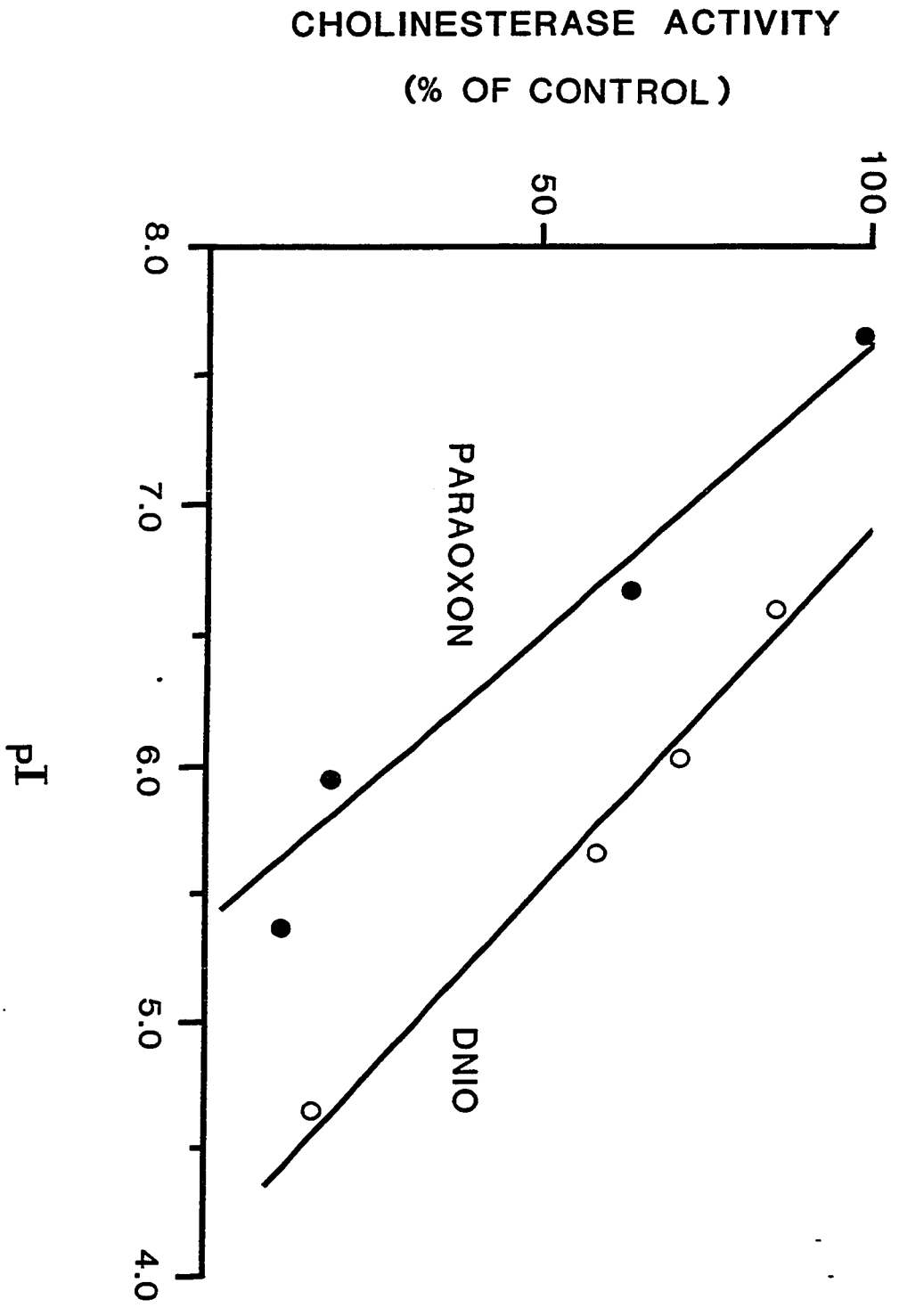

100
10
1
TIME (MIN)
6.0
5.0
4.0
3.0
pI
PARAOXON
DNIO
ISOFENPHOS OXON
DNI
ISOFENPHOS

Figure 5. Cholinesterase activity *in vitro* versus inhibitor concentration

The mean control rate equals 100% and was 72 uM mg^{-1} h^{-1}


CHOLINESTERASE ACTIVITY
(% OF CONTROL)
100
50
8.0
7.0
6.0
5.0
4.0
pI
PARAOXON
DNIO

DNI were dissolved in 2.0% rather than 0.3% DMSO. However, this change did not increase cholinesterase inhibition for any of these compounds.

In Vivo Cholinesterase Assay

In contrast to the results from the in vitro assay, all five compounds tested in the in vivo assay showed a dose-dependent inhibition of cholinesterase. Paraoxon and DNIO were the most potent, with I_{50} values of 3.1×10^{-6} M and 3.6×10^{-6} M, respectively. Lower potencies were obtained with isofenphos oxon ($I_{50} = 2.3 \times 10^{-5}$ M), DNI ($I_{50} = 3.3 \times 10^{-5}$ M), and isofenphos ($I_{50} = 9.8 \times 10^{-3}$ M). Comparison of these I_{50} values with the I_{50} value of paraoxon (Table 1) indicated that the inhibitory effectiveness of DNIO was similar to paraoxon, whereas isofenphos, isofenphos oxon and DNI were much less active.

To inhibit the action of enzyme systems that may be involved in the metabolism of the five compounds, the VNC was pretreated with SKF 525A before addition of inhibitor. Table 1 shows the I_{50} values for each compound after SKF 525A pretreatment. Although cholinesterase inhibition by paraoxon and isofenphos was slightly increased, the potencies of DNIO and isofenphos oxon were decreased. Comparison of I_{50} values of each compound relative to paraoxon indicated SKF 525A pretreatment decreased DNIO relative potency from 86% (without SKF 525A) to 4% (with SKF 525A). Inhibition by isofenphos oxon was also substantially decreased.

EC_{15} Values and Cholinesterase Activity

Comparisons between the dose-response relationships for electrophysiological effects (Figure 4) and cholinesterase inhibition in

Table 1. In Vivo Cholinesterase Inhibition

No SKF 525A Treatment

Compound	I_{50} (M)	95% CI (M)	Relative Activity (Paraoxon = 100)
Paraoxon	3.1×10^{-6}	$1.02 \times 10^{-6} - 8.91 \times 10^{-6}$	100
DNIO	3.6×10^{-5}	$1.74 \times 10^{-5} - 7.59 \times 10^{-5}$	86
Isofenphos oxon	2.3×10^{-5}	$1.70 \times 10^{-5} - 3.16 \times 10^{-5}$	14
DNI	3.3×10^{-5}	$1.86 \times 10^{-5} -$ [b] 5.75×10^{-5}	9
Isofenphos	$\sim 9.8 \times 10^{-3}$	- [b]	<1

With SKF 525A Treatment

Compound	I_{50} (M)	95% CI (M)	Relative Activity (Paraoxon = 100)
Paraoxon	1.6×10^{-6}	$8.32 \times 10^{-7} - 2.82 \times 10^{-6}$	100
DNIO	4.0×10^{-5}	$1.88 \times 10^{-5} -$ [b] 8.32×10^{-5}	4
Isofenphos oxon	$\sim 7.4 \times 10^{-4}$	- [b]	<1
DNI	- [a]	- [a]	- [a]
Isofenphos	$\sim 2.6 \times 10^{-3}$	- [b]	<1

[a] Values could not be determined; there was no dose-dependent inhibition of cholinesterase.

[b] Exact I_{50} values could not be determined; consequently 95% CIs were not calculated.

vivo permitted the estimation of the expected levels of cholinesterase inhibition at the onset of bursting. Inasmuch as all incubation times for the cholinesterase assays in vivo were 15 min, the corresponding effective concentration of inhibitor that induced bursting after 15 min (designated the EC_{15} value) was determined by extrapolation from Figure 4. These EC_{15} values were then used, along with I_{50} values (Table 1) and plots of cholinesterase activity in vivo versus inhibitor concentration, to calculate the percentages of cholinesterase activity corresponding to the EC_{15} value for each compound. Cholinesterase activities at the EC_{15} value for each compound were similar, ranging from 27% to 42% of control activity (Table 2).

Table 2. Comparison of $EC_{15\ min}$ and Cholinesterase Activity

Compound	$EC_{15\ min}$ (M)	Cholinesterase Activity (% of control)
Paraoxon	5.37×10^{-6}	42
DNIO	3.16×10^{-5}	27
Isofenphos oxon	7.94×10^{-5}	36
DNI	1.40×10^{-4}	36

DISCUSSION

Several studies have shown that the application of OPs to the cockroach VNC causes both abnormal neuronal discharges and cholinesterase inhibition. For example, demeton and methyldemeton (Narahashi and Yamasaki, 1960) induced spontaneous activity, caused prolonged cercal afferent-GI after-discharges, and eventually blocked synaptic transmission. Cholinesterase activity was reduced to 50% at the time that increased spontaneous activity occurred. It was concluded that cholinesterase inhibition was responsible for the induced electrophysiological activity. In a similar study using diazoxon (Burt et al., 1966), cholinesterase activity in the sixth abdominal ganglion was correlated with the time to block of the cercal-GI synapse. Cholinesterase activity steadily decreased following diazoxon treatment and was about one-half normal when the synapse was blocked.

Although there have been no studies correlating the effects of phosphoramidothionates or metabolites on both electrophysiological and cholinesterase activity, previous studies have shown that phosphoramidothiolates can inhibit cholinesterase acitvity *in vivo*, but are relatively poor inhibitors *in vitro*. For example, topically applied methamidophos (Monitor^{R}) is highly toxic to *Musca domestica* L. (LD_{50} = 1.3 ug/g), but was only a moderately active inhibitor of housefly head cholinesterase (I_{50} = 3.9 X 10^{-5} M) (Quistad et al., 1970). When compared with paraoxon, methamidophos was a poor inhibitor *in vitro* of acetylcholinesterase obtained from the electric eel, the tobacco budworm

Heliothis virescens (F.), and the boll weevil Anthonomus grandis grandis (Boheman) (Rose and Sparks, 1984). Two hypotheses have been offered to explain the poor correlation between methamidophos toxicity and cholinesterase inhibition in vitro. The toxicity of methamidophos may be related to its stability in vivo, resulting in gradual internal accumulation and subsequent cholinesterase inhibition (Khasawinah et al., 1978; Magee, 1982; Rose and Sparks, 1984); alternatively, activation of methamidophos to a more toxic form may be required for cholinesterase inhibition (Eto et al., 1977). The hypothesis that methamidophos may be oxidatively activated has received some support (Thompson and Fukuto, 1982), but another study provided evidence against this view (Wing et al., 1983). Recently, kinetic studies have shown that phosphorylation rates of phosphoramidates contributed significantly toward acetylcholinesterase and cholinesterase inhibition (Singh, 1985).

The spontaneous bursting activity (Figure 2) induced by the phosphoramidate isofenphos and its metabolites appeared indistinguishable from the bursting induced by treatment with parathion (Colhoun, 1960; Yamasaki and Narahashi, 1960). Intracellular recordings obtained from ventral GIs during treatment with the cholinesterase inhibitor, paraoxon (Figure 3), indicated that GI1 and GI2 were among the units exhibiting this spontaneous bursting activity. Because these neurons receive cholinergic input from cercal afferent fibers within the sixth abdominal ganglion (Sattelle, 1980; Sattelle and Harrow, 1983), it is reasonable to hypothesize that observed bursting in GIs resulted from cholinesterase inhibition. Results from the in vivo cholinesterase assays (Table 1)

support this hypothesis. For example, the ordering in potency of the three metabolites was the same with respect to induction of bursting activity (Figure 4) and cholinesterase inhibition *in vivo* (Table 1). Furthermore, when the percentage of cholinesterase activity was determined at the respective EC_{15} value for each compound, the corresponding activity level was similar for all compounds (Table 2), suggesting that these compounds share a similar mode of action.

Previous studies using isofenphos have shown that the toxicity of isofenphos involves its metabolites. For example, the incubation of isofenphos with a rat liver microsomal system increased the inhibition of bovine serum acetylcholinesterase, suggesting oxidative bioactivation (Ueji and Tomizawa, 1984). In addition, a recent study by Chow et al. (1986) has shown that DNIO may be involved in causing delayed neuropathy. The present study provides several lines of evidence that cholinesterase inhibition by isofenphos requires bioactivation. First, cholinesterase assays showed that isofenphos was the least potent compound in inhibiting cholinesterase both *in vitro* and *in vivo* (Table 1) and required a long time to induce electrophysiological effects (Figure 4). Second, three of the known metabolites of isofenphos were relatively potent inducers of bursting activity and inhibitors of cholinesterase, suggesting that these bioactivated compounds may be metabolically closer to a highly active anticholinesterase agent than isofenphos. Although the most potent metabolite, DNIO, and paraoxon have similar I_{50} values *in vivo* (10×10^{-6} M), DNIO was about one order of magnitude less potent than paraoxon *in vitro*. One possibility for these differences is that further metabolism

of DNIO was necessary. Evidence for this was also provided by the experiments with SKF 525A, an inhibitor of MFO systems. When DNIO, isofenphos oxon, and DNI were added to preparations pretreated with SKF 525A, these compounds were less active (Table 1) in inhibiting cholinesterase.

Results from the present study indicate that the mechanism of action for isofenphos involves bioactivation and subsequent cholinesterase inhibition by metabolites. DNIO, a product resulting from two oxidation steps, is the most effective inhibitor of cholinesterase. However, its activity is reduced by an MFO inhibitor, suggesting that a further oxidative activation step may be necessary for optimal potency. Additional research will be necessary to investigate this possibility.

REFERENCES

Burt, P. E., Gregory, G. E., and F. M. Molloy. 1966. A histochemical and electrophysiological study of the action of diazoxon on cholinesterase activity and nerve conduction in ganglia of the cockroach Periplaneta americana L. Ann. Appl. Biol. 58:341-354.

Chow, E., J. N. Seiber, and B. W. Wilson. 1986. Isofenphos and an in vitro activation assay for delayed neuropathic potential. Toxicol. Appl. Pharmacol. 83:178-183.

Colhoun, E. H. 1960. Acetylcholine in Periplaneta americana L. IV. The significance of esterase inhibition in intoxication, acetylcholine levels, and nervous conduction. Can. J. Biochem. Physiol. 38:1363-1376.

Corbett, J. R., Wright, K., and A. C. Baillie, eds. 1984. The Biochemical Mode of Action of Pesticides, second edition. Academic Press, London, New York.

Daley, D. L., Vardi, N. G., Appignani, B., and J. M. Camhi. 1981. Morphology of the giant interneurons and cercal nerve projections of the American cockroach. J. Comp. Neurol. 196:41-52.

Diggle, W. M., and J. C. Gage. 1951a. Cholinesterase inhibition in vitro by O,O-diethyl O-p-nitrophenyl thiophosphate (Parathion, E605). Biochem. J. 49:491-494.

Diggle, W. M., and J. C. Gage. 1951b. Cholinesterase inhibition by parathion in vivo. Nature 168:998.

Edwards, A. 1980. Cholinesterase activity in the cockroach central nervous system. Insect Biochem. 10:387-392.

Ellman, G. L., Courtney, K. D., Andres, V., Jr., and R. M. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7:88-95.

Eto, M., Okaabe, S., Ozoe, Y., and K. Maekawa. 1977. Oxidative activation of O,S-dimethyl phosphoramidothiolate. Pestic. Biochem. Physiol. 7:367-377.

Heppner, T. J., Coats, J. R., and C. D. Drewes. September, 1985. Relative potency of paraoxon, isofenphos and three of its metabolites in induction of activity within the cockroach ventral nerve cord. 190th National meeting of the American Chemical Society, Chicago, Illinois. AGRO 130.

Hsin, C.-Y., and J. R. Coats. (in press). Metabolism of isofenphos in southern corn rootworm. Pestic. Biochem. Physiol.

Khasawinah, A. M. A., March, R. B., and T. R. Fukuto. 1978. Insecticidal properties, antiesterase activities, and metabolism of methamidophos. Pestic. Biochem. Physiol. 9:211-221.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

Magee, P. S. 1982. Structure-activity relationships in phosphoramidothioates. Pages 101-161 in J. R. Coats, ed. Insecticide Mode of Action. Academic Press, New York.

Manulis, S., Ishaaya, I. and A. S. Perry. 1981. Acetylcholinesterase of Aphis citricola: Properties and significance in determining toxicity of systemic organophosphorus and carbamate compounds. Pestic. Biochem. Physiol. 15:267-274.

Metcalf, R. L., and R. B. March. 1953a. The isomerization of organic thionophosphate insecticides. J. Econ. Entomol. 46:288-294.

Metcalf, R. L., and R. B. March. 1953b. Further studies on the mode of action of organic thionophosphate insecticides. Ann. Entomol. Soc. Am. 46:63-74.

Narahashi, T., and T. Yamasaki. 1960. Nervous and cholinesterase activities in the cockroach as affected by demeton and methyl demeton. Jpn. J. Appl. Entomol. Zool. 4:64-69.

Neely, W. B., and W. K. Whitney. 1968. Statistical analysis of insecticidal activity in a series of phosphoramidates. J. Agric. Food Chem. 16:571-573.

Quistad, G. B., Fukuto, T. R., and R. L. Metcalf. 1970. Insecticidal, anticholinesterase, and hydrolytic properties of phosphoramidothiolates. J. Agric. Food Chem. 18:189-194.

Rose, R. L., and T. C. Sparks. 1984. Acephate toxicity, metabolism, and anticholinesterase activity in Heliothis virescens (F.) and Anthonomus grandis grandis (Boheman). Pestic. Biochem. Physiol. 22:69-77.

Sanborn, J. R., and T. R. Fukuto. 1972. Insecticidal, anticholinesterase, and hydrolytic properties of S-aryl phosphoramidothioates. J. Agric. Food Chem. 20:926-930.

Sattelle, D. B. 1980. Acetylcholine receptors of insects. Pages 215-315 in M. J. Berridge, J. E. Treherne and V. B. Wigglesworth, eds. Advances in Insect Physiology. Academic Press, New York.

Sattelle, D. B., and I. D. Harrow. 1983. α-Bungarotoxin blocks excitatory synaptic transmission between cercal sensory neurones and giant interneurone 2 of the cockroach Periplaneta americana. J. Exp. Biol. 107:473-489.

Singh, A. K. 1985. Kinetic analysis of inhibition of brain and red blood cell acetylcholinesterase and plasma cholinesterase by acephate or methamidophos. Toxicol. Appl. Pharmacol. 81:302-309.

Smissaert, H. R. 1976. Reactivity of a critical sulfhydryl group of the acetylcholinesterase from aphids (Myzus persicae). Pestic. Biochem. Physiol. 6:215-222.

Stegwee, D. 1951. Studies on cholinesterase in insects. Physiol. Comp. Oecol. 2:241-247.

Thompson, C. M., and T. R. Fukuto. 1982. Mechanism of cholinesterase inhibition by methamidophos. J. Agric. Food Chem. 30:282-284.

Ueji, M., and C. Tomizawa. 1984. Bioactivation of N-alkyl substituted phosphoramidothioate insecticides. J. Pestic. Sci. 9:675-680.

Wing, K. D., Glickman, A. H., and J. E. Casida. 1983. Oxidative bioactivation of S-alkyl phosphorothiolate pesticides: Stereospecificity of profenfos insecticide activation. Science 219:63-65.

Yamasaki, T., and T. Narahashi. 1960. Synaptic transmission in the last abdominal ganglion of the cockroach. J. Insect Physiol. 4:1-13.

Zahave, M., Tahori, A. S., and F. Klimer. 1972. An acetylcholinesterase sensitive to sulfhydryl inhibitors. Biochim. Biophys. Acta 276:577-583.

PART II. AN ELECTROPHYSIOLOGICAL STUDY OF THE EFFECTS OF PARAOXON ON THE CERCAL-TO-GIANT INTERNEURON SYNAPSE IN THE AMERICAN COCKROACH, PERIPLANETA AMERICANA

INTRODUCTION

The cercal-to-giant interneuron pathway in the cockroach has been extensively studied (for review see Ritzmann, 1984) and is therefore a useful model for studying the cellular and biochemical modes of action of neurotoxicants. Cercal afferent fibers, arising from wind-sensitive receptors located on the cerci, synapse monosynaptically onto giant interneurons (GIs). Results from numerous pharmacological studies have shown that this synapse is cholinergic for at least some of the GIs (Shankland et al., 1971; Callec, 1974; Sattelle et al., 1976; Sattelle, 1978; Sattelle, 1980). Studies using modifications of the oil-gap technique, have shown that the carbamate eserine potentiates the effect of acetylcholine application to the sixth abdominal ganglion (A6) and also prolongs the EPSP at the cercal-to-GI synapse (Callec, 1974). The neurotoxic effects of organophosphate insecticides (OPs) on the cercal-to-GI pathway were first documented in experiments by Roeder et al. (1947). In this study, application of the OP diisopropylfluorophosphate (DFP) to A6 (6×10^{-4} M), induced prolonged afterdischarges of GIs in response to cercal stimulation. Subsequently, the cercal-to-GI synapse was blocked for 30-60 seconds. Synapse function then began alternating between the hyperexcitable and blocked states for 2-3 hours. The enhanced postsynaptic response was attributed to increased levels of acetylcholine as a result of cholinesterase inhibition. Subsequent studies with various OPs (Yamasaki and Narahashi, 1958; Colhoun, 1960; Yamasaki and Narahashi, 1960; Narahashi and

Yamasaki, 1960; Burt et al., 1966; Heppner et al., in press) also showed correlations between electrical activity in the ventral nerve cord (VNC) and cholinesterase inhibition. One limitation in understanding the mode of action of OPs on the insect nervous system is the lack of intracellular microelectrode studies of GI activity during OP intoxication.

The purpose of this study was to describe the intracellular and extracellular electrophysiological effects of the OP paraoxon on the cercal afferent-GI synapse in the American cockroach.

METHODS AND MATERIALS

Preparation

Cultures of Periplaneta americana were raised on Purina^{R} dog chow and water at a temperature of 27^{o}C. Adult male cockroaches were selected at random and pinned, dorsal side up, in a small trough cut into a Sylgaard^{R} dish. The VNC was exposed by removing the dorsal cuticle and gut. Care was taken to ensure that large tracheae supplying the VNC remained intact. A 0.5 ml aliquot of saline (Sattelle and Harrow, 1983) was then placed in the body cavity and completely covered the VNC. For experiments using acetylcholine (acetylcholine chloride, Sigma) the dorsal neural sheath of A6 was removed using microscissors and sharpened forceps.

Microelectrode penetration of ventral giant interneuron (vGI) cell bodies in A6 was facilitated by placing several small crystals of protease (Sigma) on the neural sheath overlying the cell bodies. After approximately 1.5 min the ganglion was rinsed repeatedly with saline. Before penetration, a ganglion platform was placed beneath the VNC and a glass light guide was used to focus light on the preparation.

Solutions

Ethyl paraoxon was checked for purity using thin-layer chromatography. For most experiments 1-2 drops of a 20 ppm (7.3×10^{-5} M) paraoxon solution (paraoxon dissolved in saline) were added to the bath after normal electrophysiological responses were obtained from the preparation. Final bath concentration of paraoxon was approximately

5.5×10^{-6} M. For some experiments final concentrations of 3.6×10^{-7} M were used.

Electrophysiology

For most experiments extracellular recording electrodes were placed under the VNC between the fifth abdominal ganglion (A5) and A6, and an intracellular microelectrode filled with 3 M KCl was inserted into a GI just anterior to A6. In some experiments an intracellular microelectrode was inserted into vGI cell bodies within A6 (Daley et al., 1981; Harrow et al., 1980; Harris and Smyth, 1971). The microelectrode was presumed to be inserted into a vGI cell body when cercal afferent stimulation simultaneously evoked a short latency intracellular action potential and a large amplitude extracellular spike in the connectives. Afferent stimulation was by a pair of 50 uM silver wires inserted into the cut end of a cercus. Extracellular recordings were amplified by a Grass P15 preamplifier and displayed on a Tektronix 5111 storage oscilloscope. Intracellular recordings were obtained using a WPI model M701 or model KS-700 microprobe system. All recordings were stored on magnetic tape for later analysis.

Upon completion of some experiments, the fluorescent dye lucifer yellow, dissolved in 1 M LiCl, was iontophoresed into the impaled axon. Identification of the GI was based on cell body location and dendritic branching patterns within A6 (Daley et al., 1981; Harrow et al., 1980). In cases in which dendritic branches were inadequately filled, cross sections of A5 were obtained with a cryostat. Identification was then

based on the position of the filled GI axon in A5 (Camhi, 1976; Harrow et al., 1980; Daley et al., 1981).

RESULTS

Paraoxon Effects

The application of paraoxon (5.5×10^{-6} M) to the preparation induced high-frequency bursting activity within five min in many units within the A5-A6 connective (Fig 1A). Burst activity was first evident as high-frequency repetitive firing in low-voltage units (ca. 35 uV); then progressively larger units (up to 650 uV) were recruited within a burst. Often the activity of low and high-voltage units overlapped. Firing of high-voltage units was followed by a quiescent period. Then after approximately 30-120 s, another similar bursting episode occurred. This cyclical bursting persisted for at least one hour.

To examine activity in individual units during paraoxon treatment, intracellular microelectrodes were inserted into the vGI axons (GI1 or GI2) just anterior to A6. The membrane potential (Em) before the addition of paraoxon was -62.3 mV $\pm$ 7.09 SD; n = 5. The addition of 3.6×10^{-7} M paraoxon to the preparation did not change Em within 30 min. However, at higher concentrations (5.5×10^{-6} M) significant changes occurred within a few minutes after application. The first sign of a paraoxon effect was a rapid depolarization that eventually triggered repetitive, overshooting spikes in the axon (Fig 2A). The onset of spiking occurred when the axon was depolarized by a mean value of 2.2 mV $\pm$ 1.09 SD; n = 5. As depolarization continued, spike frequency increased to greater than 300 Hz.

During high-frequency firing, individual spikes were often preceded

Figure 1. Long-term extracellular recording from A5-A6

A) Approximately 10 min after the addition of paraoxon, cyclical bursting began. B) Prolonged firing of many units occurred after the addition of 10^{-2} M acetylcholine

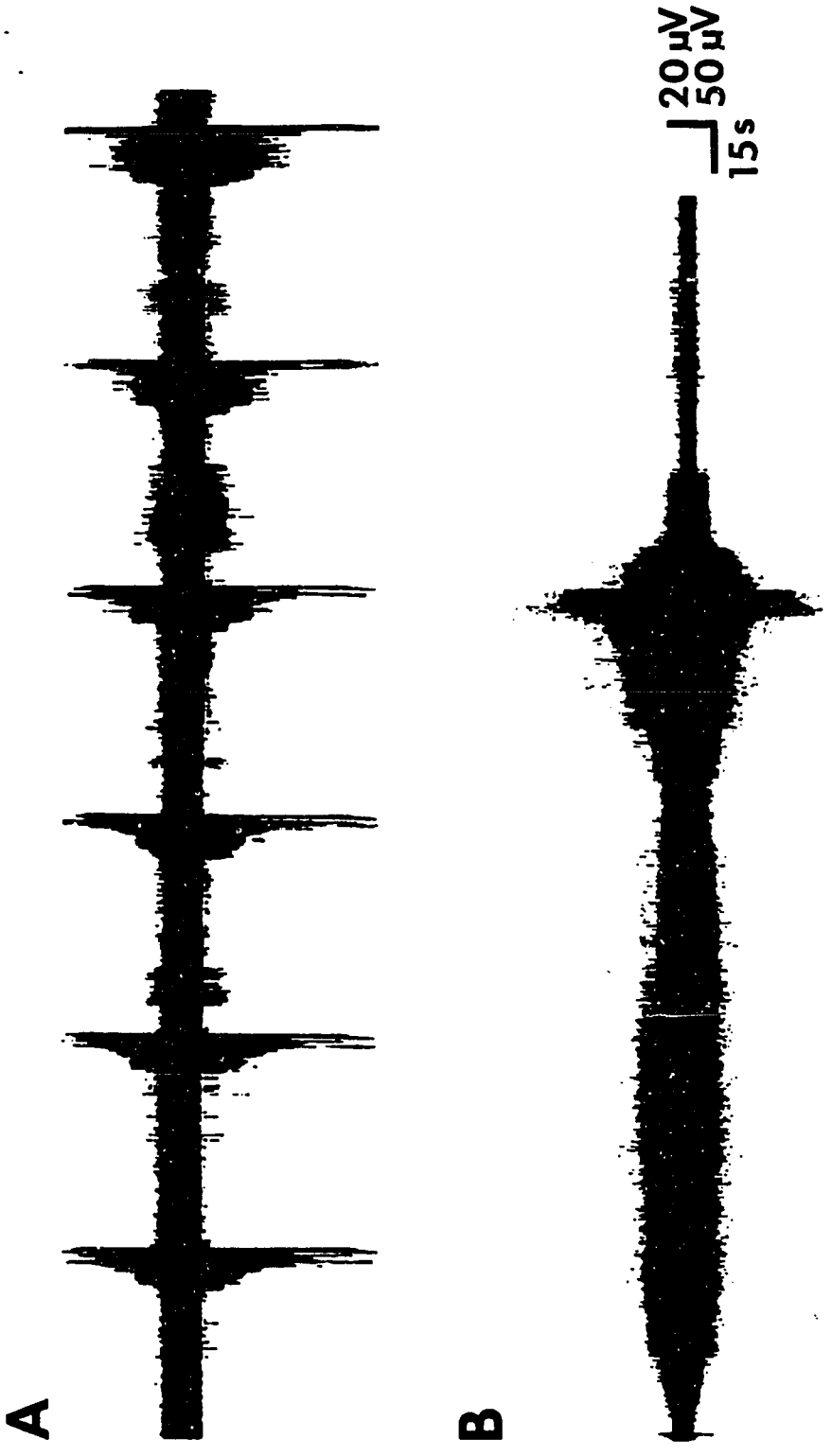
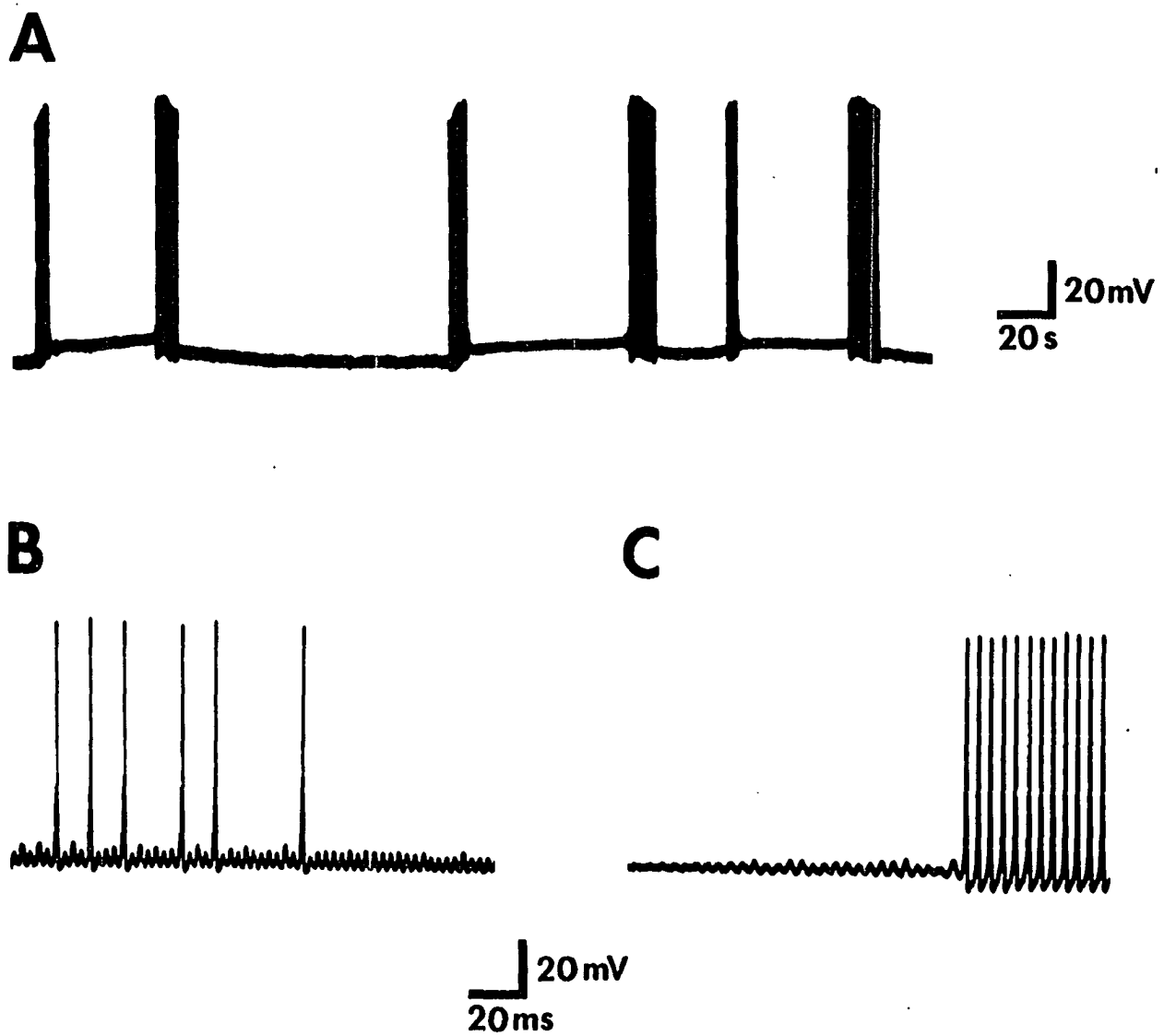

A
B
20 µV
50 µV
15s

Figure 2. Long-term intracellular recording from a vGI after the onset of paraoxon-induced cyclical bursting

A) Bursts of overshooting spikes were superimposed on the depolarizing and repolarizing shifts in membrane potential. B) The end of the first depolarizing burst in A is shown at a fast sweep speed. Overshooting spikes intermittently failed, leaving small amplitude spikes. C) The beginning of the first repolarizing burst in A is shown at a fast sweep speed. Small amplitude spikes appear to evoke overshooting spikes

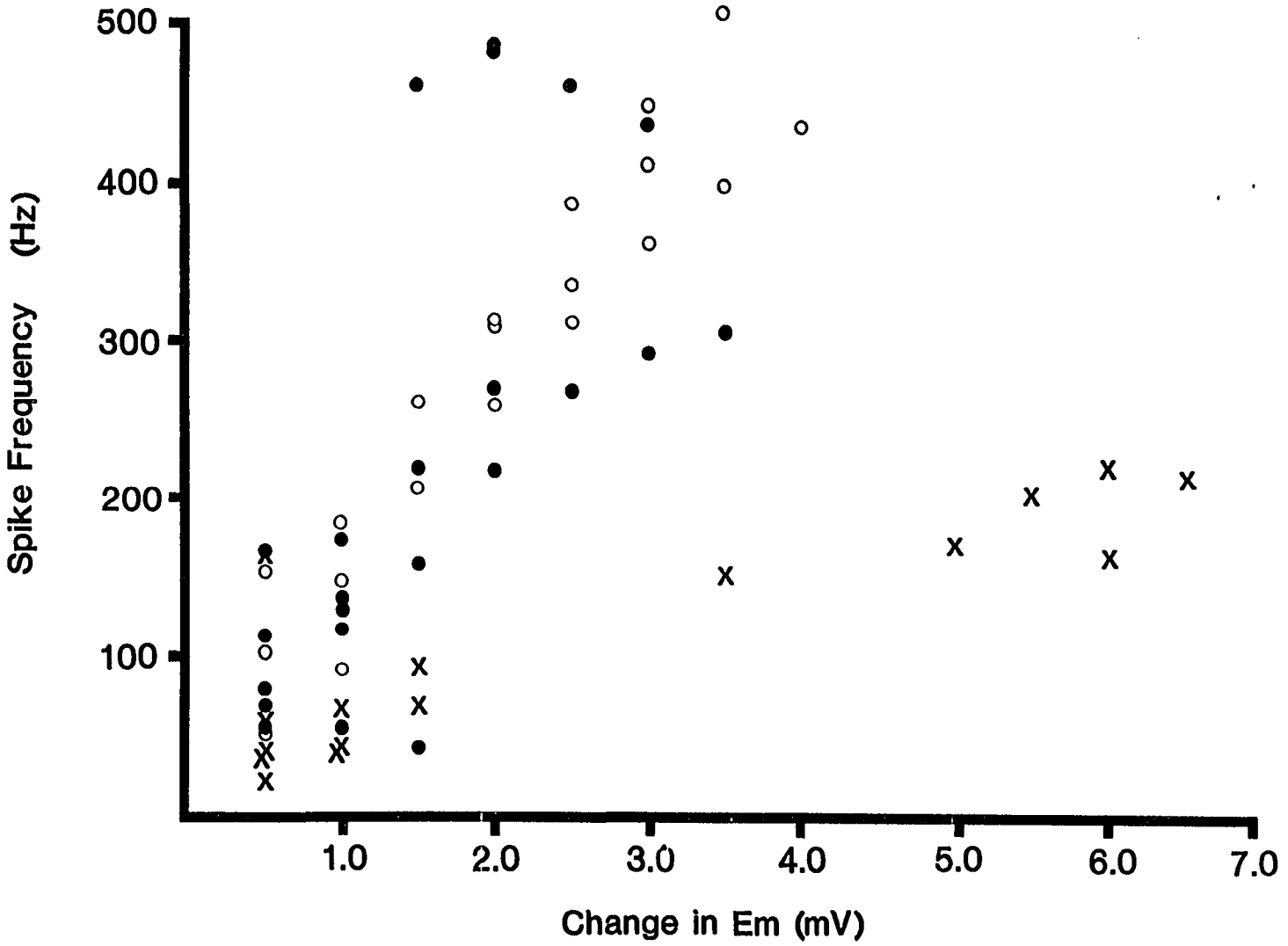

A
20mV
20s
B
C
20mV
20ms

by a small prepotential of 2-8 mV. As the effects of paraoxon treatment continued, the overshooting spikes often failed intermittently, with only the spike-like prepotentials persisting (Fig 2B). The frequency of these small spikes continued to increase (up to > 400 Hz) as membrane depolarization continued.

The relationship between the level of membrane depolarization and the frequency of small and overshooting spikes is shown in Fig 3. As the axonal depolarization continued, the amplitude of the small spikes decreased until eventually the spikes were difficult to distinguish from background noise. Thereafter, a plateau of depolarization was maintained for several seconds to several minutes. The plateau ended with the beginning of membrane repolarization and onset of high-frequency, small-amplitude spiking, the latter appearing identical to that seen just before the plateau (Fig 2B, C). As repolarization continued, the amplitude and frequency of small spikes decreased. Then normal overshooting spikes were intermittently superimposed on the regularly-occurring small spikes. Eventually the frequency of overshooting spikes became regular, and small spikes were no longer apparent. Presumably the small spikes were still in phase with (but at least partially obscured by) the overshooting spikes, because shoulder-like prepotentials occasionally preceded overshooting spikes. As the membrane potential neared the resting level, overshooting spikes stopped. This pattern of depolarization and repolarization, along with the accompanying spiking (Fig 2A), was maintained for more than 30 min. Throughout most of the paraoxon treatment the axon appeared to be in the

Figure 3. Scatter plot of spike frequency (overshooting and small spikes) versus membrane potential during depolarizing bursts

Data points were obtained from axonal (●) and cell body (O) recordings during paraoxon treatment, and axonal recordings (X) during acetylcholine treatment


500
400
300
200
100
Spike Frequency (Hz)
1.0
2.0
3.0
4.0
5.0
6.0
7.0
Change in Em (mV)

repolarized, rather than depolarized or bursting state.

To examine the origin of GI spike initiation during paraoxon treatment, a portion of the abdominal VNC was isolated by ligation at both A1-A2 and A4-A5. Intracellular recordings from the GI axons between these ligations showed no depolarization or spiking activity during paraoxon treatment (n = 3). Extracellular activity during treatment showed some low-voltage spiking within the isolated region, but there was no evidence of high-voltage spikes. However, extracellular recordings from the connective posterior to the ligation at A4-A5, but just anterior to A6, indicated high-frequency, high-voltage spikes (Fig 4). Therefore, the initiation of the paraoxon-induced GI spiking appeared to be within or near A6.

To more precisely localize these effects, intracellular recordings were obtained from vGI cell bodies during paraoxon treatment (resting Em = -62.2 mV $\pm$ 2.08 SD; n = 3). These recordings showed periods of alternating depolarization and repolarization, as in axon recordings. Depolarization was accompanied by initiation of small amplitude spikes (up to 7.0 mV), whose frequency increased in relation to the level of depolarization. Initially the small spikes were in phase with high-voltage spikes recorded extracellularly in the connectives. This suggests that the spikes recorded at these two locations have a common origin, presumably at some spike-initiating region in A6. However, as depolarization and small spike frequency increased, the high-voltage spikes in the connective failed, but not the small spikes in the cell body continued (Fig 5). This suggests that GI spike initiation continues

Figure 4. Simultaneous extracellular recordings during paraoxon treatment between two VNC ligations (lower trace), and a more posterior region adjacent to A6 (upper trace)

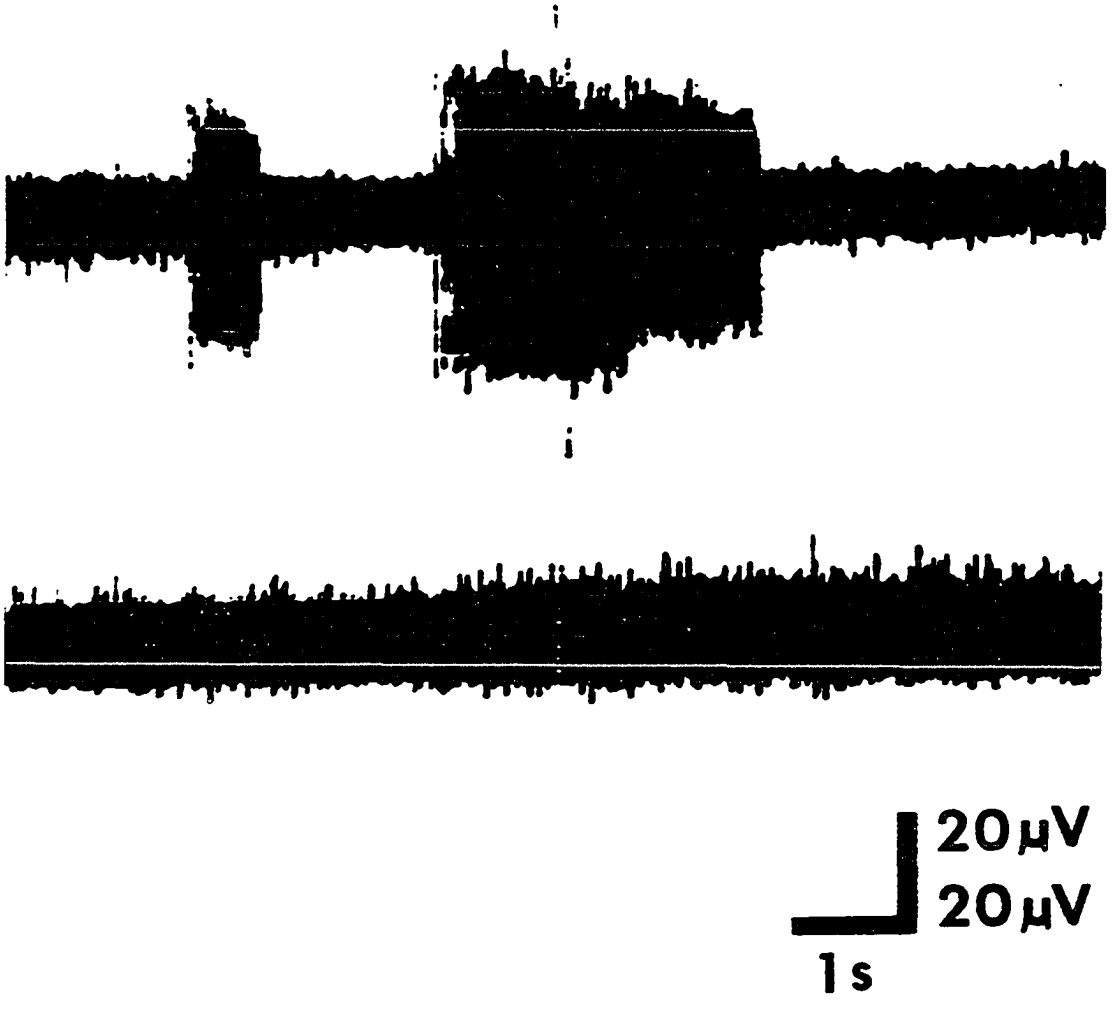
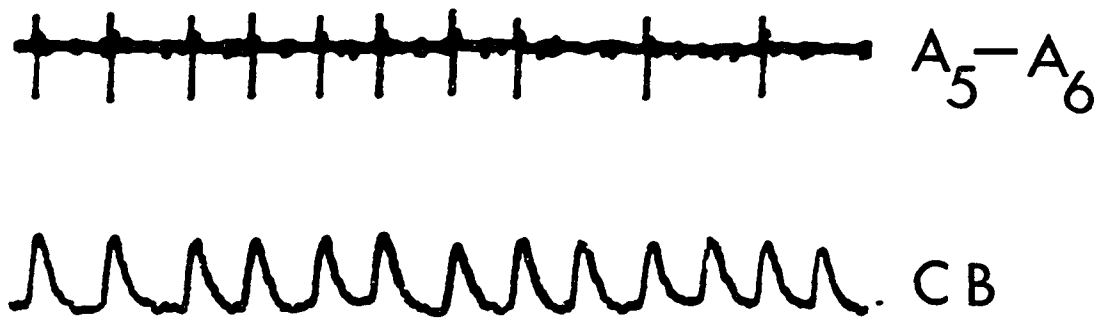

20μV
20μV
1s

Figure 5. Simultaneous recordings (upper trace, extracellular) from a vGI cell body (lower trace, intracellular) during paraoxon-induced bursting

Axonal spikes failed intermittently, but cell body spikes continued firing


A_5-A_6
CB
200 μV
20 mV
10 ms

in A6, in spite of spike conduction failure into the axon.

The possibility that known excitatory synaptic inputs (i.e., cercal sensory) to GIs were responsible for the paraoxon-induced effects was examined in two ways. First, extracellular recordings were made from the cercal afferent pathways before treatment, and during paraoxon-induced GI spiking. Such recordings showed that activity in the cercal sensory nerve throughout the entire treatment period was indistinguishable from the normal background level seen before treatment (Fig 6). Second, evidence that cercal inputs were not involved in the paraoxon-induced activity was obtained by removing both cerci and cercal sensory nerves 12-14 days prior to paraoxon treatment. This procedure is known to cause degeneration of cercal presynaptic terminals (Farley and Milburn, 1969). In each of the deafferented preparations (n = 7) paraoxon still induced typical high-frequency bursts (Fig 7). Together these results suggest that cercal inputs do not contribute to the generation of the depolarization plateau or spiking activity observed during paraoxon treatment.

To test whether the efficacy of cercal-to-GI synaptic transmission was impaired by paraoxon treatment in intact animals, afferent activity was evoked by electrical stimulation of the cercal nerves during paraoxon treatment. Application of 3.6×10^{-7} M paraoxon did not alter EPSP amplitude or duration within 30 min. At higher concentrations (5.5×10^{-6} M) which induced depolarization and bursting (Fig. 2A), synaptic transmission was blocked, as indicated by the failure of the presynaptic afferent volley to evoke an EPSP or spike in GIs (Fig 8). Sometimes the

Figure 6. Simultaneous recordings from the connective (middle trace, extracellular), cercal sensory nerve (lower trace, extracellular), and a GI axon (upper trace, intracellular) during a paraoxon induced burst

GI spikes were not driven by any apparent cercal activity

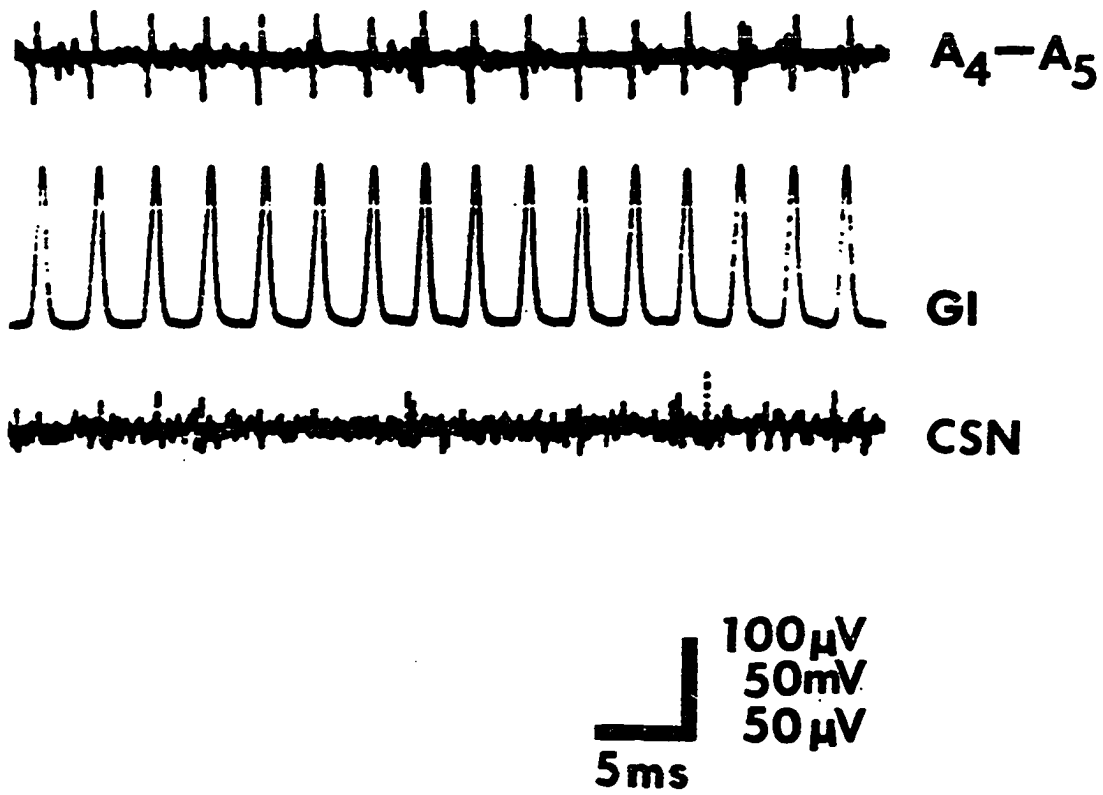

A4—A5
GI
CSN
100μV
50mV
50μV
5ms

Figure 7. Electrophysiological responses in normal and deafferented preparations during paraoxon treatment

Extracellular (upper trace) and intracellular (lower trace) recordings from a GI show similar bursting in normal and deafferented preparations

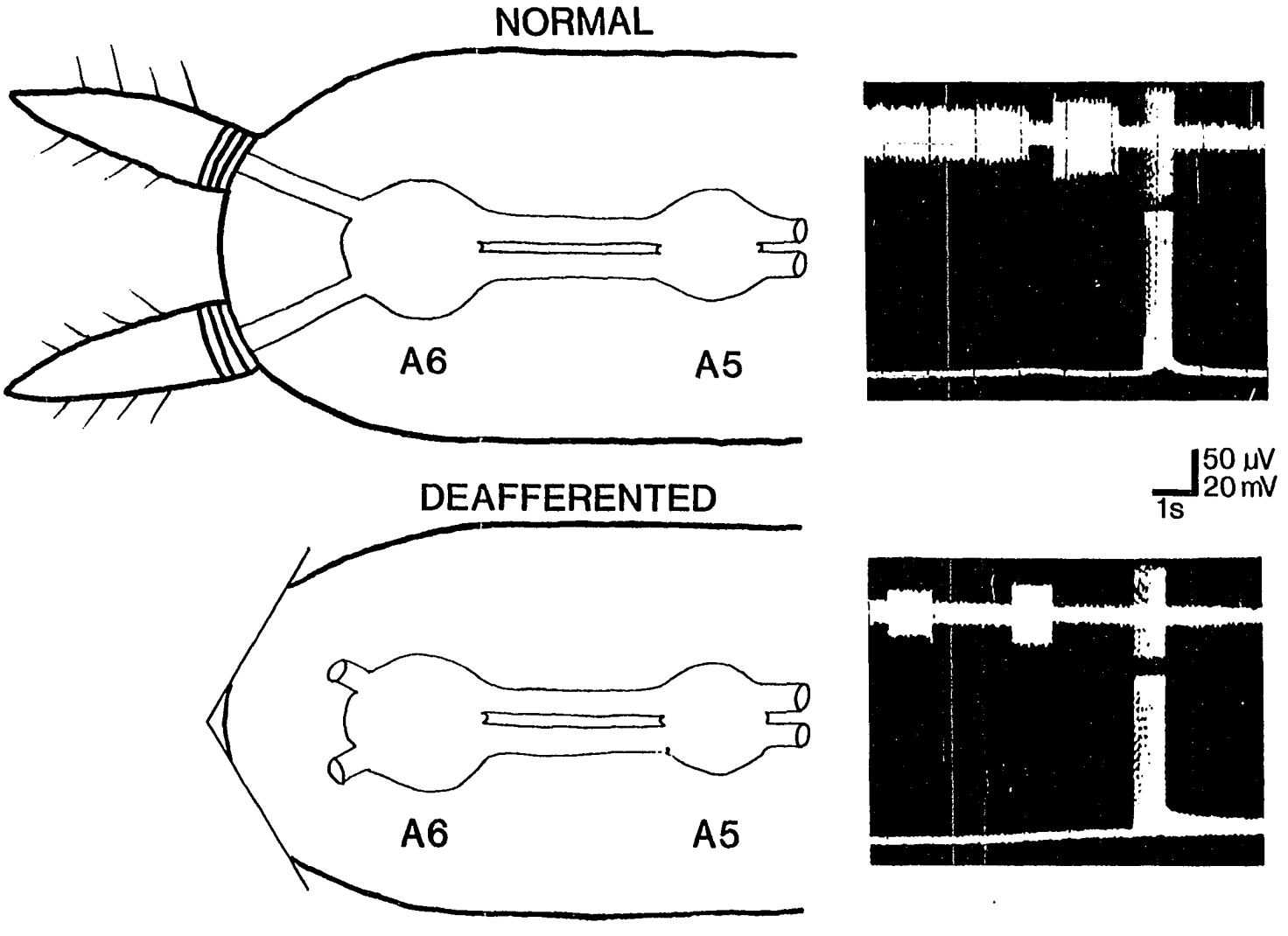
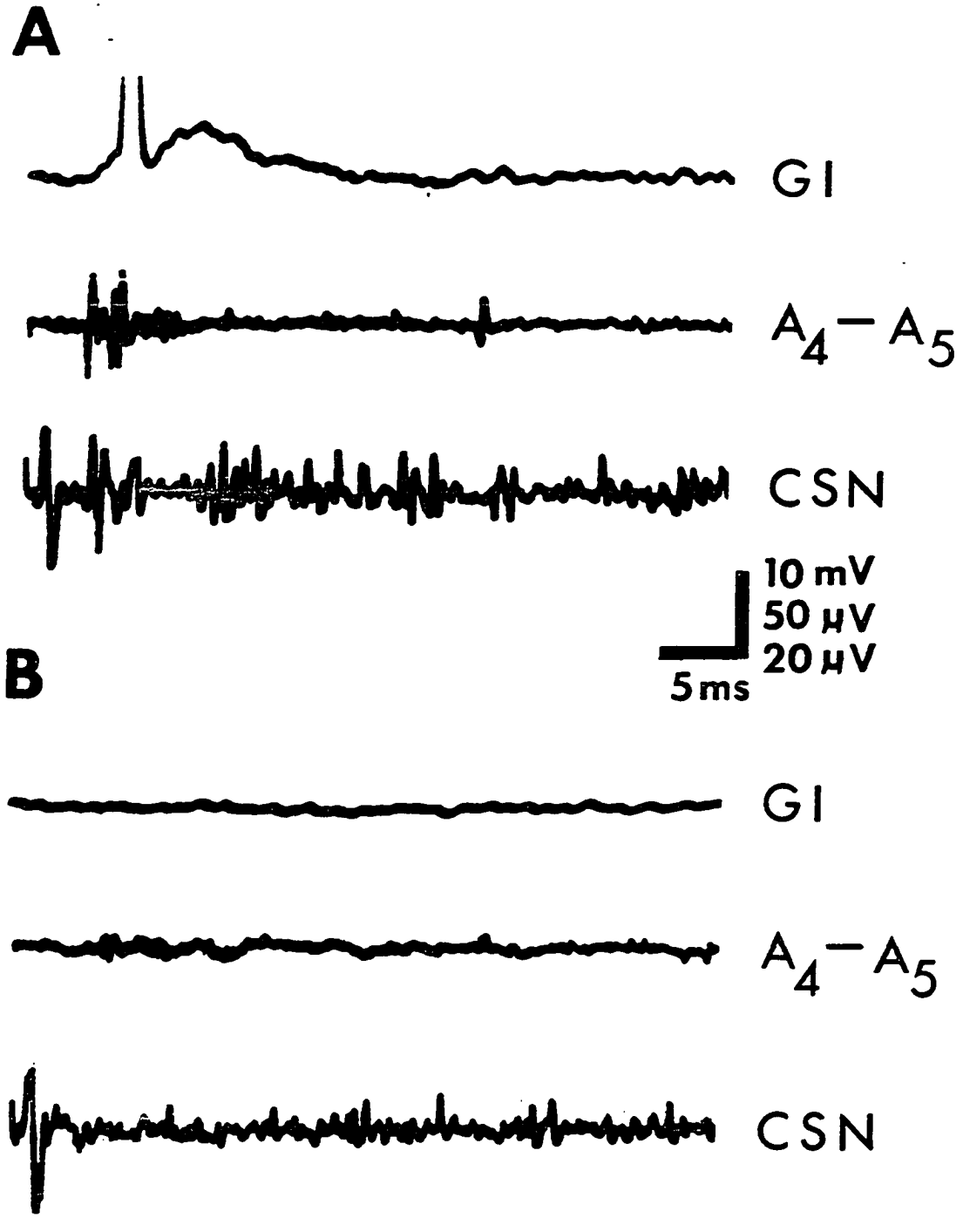

NORMAL
A6
A5
50 µV
20 mV
1s
DEAFFERENTED
A6
A5

Figure 8. Simultaneous recordings from a GI axon (upper trace, intracellular), A4-A5 connective (middle trace, extracellular), and cercal sensory nerve (lower trace, extracellular) during cercal stimulation

A) Just prior to paraoxon application an afferent volley in the cercal nerve evoked an EPSP and spike in the GI. B) Within 5 min of paraoxon addition cercal stimulation evoked a similar afferent volley, but no EPSP or spiking activity


A
GI
$A_4 - A_5$
CSN
10 mV
50 µV
20 µV
5 ms
B
GI
$A_4 - A_5$
CSN

efficacy of synaptic transmission was partially regained during the periods of repolarization between bursts, but EPSP amplitudes at these times were usually reduced to less than one-half of pretreatment values (typical pretreatment EPSP amplitude = 5 mV). The duration of the EPSP was also reduced relative to pretreatment periods.

Acetylcholine Effects

Previous studies have shown that the application of high concentrations of acetylcholine (concentrations > 10^{-3} M) to the desheathed sixth abdominal ganglion of the cockroach induced high-frequency spiking activity recorded extracellularly from the VNC (Twarog and Roeder, 1957; Yamasaki and Narahashi, 1960). To confirm and extend these results, extracellular and intracellular recordings were obtained from GIs during acetylcholine application to A6 (desheathed). Within a few minutes after application of acetylcholine (10^{-3} M) moderate increases in background firing of many units were evident in extracellular recordings from A5-A6. Higher frequencies of activity in the A5-A6 connective were induced by 10^{-2} M acetylcholine (Fig 1B). Such activity persisted continuously for up to 10 min after application. Thereafter activity was substantially diminished, except for some low-frequency tonic firing that continued up to one hour after acetylcholine application. Thus, one obvious difference between paraoxon and acetylcholine-induced activity patterns in extracellular recordings was that cyclical bursting activity was not evident during acetylcholine treatment.

Activity during acetylcholine treatment was also studied by GI penetration just anterior to A6. GIs were physiologically identified by the short latency (<2 ms) of spiking following cercal electrical stimulation and the corresponding occurrence of high-voltage spikes in extracellular recordings (A5-A6). During acetylcholine treatment (10^{-2} M) GIs depolarized gradually. This depolarization correlated with the failure of cercal stimuli to evoke an EPSP and a concomitant GI spike. As the depolarization continued, repetitive spiking occurred (Fig 9). Spikes were initially overshooting, but as spike frequency gradually increased only small spikes were evident. The small spikes were essentially identical to those seen during paraoxon treatment.

Differences between the intracellularly recorded GI activity during acetylcholine and paraoxon treatment included: a) the maximum large spike frequency was 159 Hz ($\pm$ 36.6 SD; n = 5) during acetylcholine treatment, and 318 Hz ($\pm$98.9 SD; n = 5) during paraoxon treatment; b) during acetylcholine treatment GI spiking occurred in a single, continuous train often lasting 30-60 s; in paraoxon, GI spikes occurred in repeated bursts, the burst duration ranged from 1-6 s; and c) with acetylcholine addition the GI membrane potential remained in a depolarized state after an initial high-frequency burst. In contrast, during paraoxon treatment a plateau of depolarization alternated with periods of repolarization.

interpersonal ties is linked to higher levels of IOR (Morrissey et al., 1982:57). Halpert (1982:61) concludes that the seeds for IOR are sown through informal contacts between organizations. Informal interaction is important because it facilitates work-related communication, as well as builds trust and rapport (Zeitz, 1980:79).

Formal meetings

If organizations do not communicate, they will have very little ground upon which to build any type of IOR (Mulford and Klonglan, 1982). One obvious type of IOR involves formal meetings among organizational directors. These tend to be planned in advance with a specific agenda. Compared to the other types of IOR just discussed, formal meetings involve more structured interaction. They often are called because of specific problems or issues. Many formal meetings may also happen because of mandates from higher levels of the organizations.

Conceptual Model: Application to Conservation Network

The conceptual model just presented is one level of abstraction above the conservation network being studied. The past and present organizational context, described in Chapter II, will be used to apply the conceptual model to the conservation network. Specific characteristics of the organizations and their IOR network will be discussed in relation to the components of the model.

Perceptions of the environmental context

Organizations perceive and respond to environmental conditions differently based on many factors, including their mission, structure,

Figure 9. Long-term intracellular recording from a GI after the addition of acetylcholine

Note the spiking activity and maintained depolarization

DISCUSSION

General Effects of OPs

General symptoms of OP intoxication in the cockroach VNC were first described by Roeder et al. (1947). Their study showed that treatment with DFP caused abnormal after-discharges in GIs following cercal stimulation, spontaneous bursting, and alternations between a blocked and facilitated state in the cercal afferent-to-GI synapse. Results from the present study show that symptoms accompanying treatment with the OP paraoxon include cyclical bursting of GIs (Fig 1A) and rapid synaptic block. An important feature of paraoxon-induced activity, not previously described in other studies, was the systematic recruitment of progressively larger amplitude units during each burst. The correlations of extracellular and intracellular recordings confirmed that at least some of the recruited large-amplitude units were GI1 and GI2.

Synaptic Effects

The synaptic effects of OPs have been studied in a variety of animals. Eccles and McFarlane (1949) studied the effects of anticholinesterase agents such as the carbamates, prostigmine and eserine and DFP on the curarized frog sartorius muscle. They found that these inhibitors increased the duration of the end-plate potential and, at relatively high concentrations (10^{-3} M), induced a "curare-like" decrease in the amplitude of the end-plate potential. Laskowski and Dettbarn (1979) found that paraoxon (10^{-5} - 10^{-3} M) induced an irreversible neuromuscular block in an <u>in vitro</u> rat diaphragm muscle preparation.

Their intracellular recordings showed an increases in miniature end-plate potential frequency and half-decay time. Likewise, Carlson and Dettbarn (1983) found that paraoxon, injected into rats increased giant miniature end-plate potential frequency in diaphragm preparations. These results suggest that paraoxon has both pre and postsynaptic effects at the vertebrate neuromuscular junction.

Cholinergic central synapses in insects are also affected by anticholinesterase agents. For example, Yamasaki and Narahashi (1960) examined the effects of eserine (10^{-6} M) on the cockroach cercal-to-GI synapse and noted blockage of the synapse after 30-90 min. Prior to the block, the ganglionic EPSP, as measured extracellularly, was increased in amplitude and duration. Similarly, Callec (1974), using a single fiber oil-gap technique, found that eserine, at concentrations greater than 10^{-6} M, induced an almost immediate depolarization and bursting of GIs. At lower concentrations (3.1×10^{-7} - 7.5×10^{-7} M) and within 45 min after application, eserine induced increases in both the amplitude and duration of unitary EPSPs evoked by stimulating single cercal mechanoreceptors.

Results from the present study showed that paraoxon (5.5×10^{-6} M) caused a marked reduction in the efficacy of cercal-to-GI synaptic transmission during alternating states of depolarization and repolarization. Interestingly, there was no evidence of a paraoxon induced increase in EPSP duration or amplitude at either 3.6×10^{-7} M or 5.5×10^{-6} M. This was somewhat surprising considering paraoxon is a potent inhibitor of VNC cholinesterase (Heppner et al., in press). The

failure of paraoxon (3.6×10^{-7} M) to prolong EPSP duration in GIs could be due to a number of factors. First, there may have been an insufficient time for onset of changes in EPSP parameters. Second, threshold concentrations for other possible paraoxon-induced effects (e.g., agonistic effects on cholinergic receptors) may be similar to or less than the threshold for effects on EPSP duration. Consequently, paraoxon effects on EPSP duration may have been masked or obscured by these other effects.

Conduction Block During High-frequency Bursts

One unusual feature of the paraoxon-induced spiking was the intermittent failure of overshooting spikes during high-frequency bursts. During bursts associated with membrane depolarization, overshooting spikes eventually failed as progressive depolarization occurred. However small spike-like potentials persisted, their amplitude decreasing and frequency increasing with progressive depolarization. This was followed by cessation of small spiking and attainment of a plateau of depolarization. The plateau terminated with the onset of small spikes and repolarization. During repolarization, the amplitude of small spikes increased and frequency decreased. Eventually small spikes were replaced with overshooting spikes. This sequence of events, closely resembles events seen during GI conduction failure in the third thoracic ganglia (T3) (Parnas et al., 1969). With high-frequency electrical stimulation, posteriorly conducted GI spikes failed to conduct through T3, but small electrotonically conducted spikes were detected posterior to the site of

failure. The failure of these spikes was attributed to the low safety factor for spike conduction within T3 where the GI axons form narrow isthmuses (Spira et al., 1969b). In view of the similar transition in GI diameter within A6 (Daley et al., 1981; Harrow et al., 1980) a similar explanation probably accounts for conduction failure of the GI spike in A6. In this case, spike electrogenesis in the GI was restricted to a localized region(s) within A6 and spread electrotonically to the recording site in the axon, thus accounting for the attenuated spike.

Cyclical Bursting

Intracellular recordings showed that GI bursting activity was the result of repeated alternations from depolarized to repolarized states. The cyclical nature of this bursting is difficult to explain purely on the basis of cholinesterase inhibition (for review, see Narahashi, 1971), because there is no obvious mechanism for achieving the observed alternations in membrane potential.

One mechanism that could account for the changes in membrane potential and cyclical bursting in GIs is intermittent activation of excitatory synaptic inputs onto GIs. Several lines of evidence suggest that such input originates in A6, but does not involve the cercal afferent fibers, the only known excitatory inputs onto GIs (for review see Ritzmann, 1984). First, experiments in which the connectives between A4-A5 were ligated showed that bursting originated in A6. Second, recordings from the cercal sensory nerves during high-frequency bursts showed no detectable change in activity when compared to pretreatment

activity levels. Third, the removal of both cerci and cercal sensory nerves 12-14 days prior to treatment did not prevent or diminish the cyclical bursting response to paraoxon. Assuming that non-cercal excitatory inputs to GIs existed in A6, such inputs would necessarily be intermittent, to account for the cyclical nature of bursting.

Another possibility is that GIs generate cyclical activity independent of synaptic input, either by paraoxon-induced destabilization of membrane potential (e.g., agonistic effects on receptors), or by activity-dependent increases in extracellular K^+ concentration. The latter mechanism was suggested by Spira et al. (1976) to account for membrane depolarization seen during electrically induced high-frequency firing of GIs. This phenomenon could explain the apparent sequential recruitment of various units during paraoxon-induced bursts, assuming that extracellular spaces were shared by adjacent neurones. However, this mechanism would not explain how the initial conduction of high-frequency bursts or membrane repolarizations occurred.

In conclusion, intracellular recordings from GIs during paraoxon intoxication were characterized by blocking of cercal-to-giant synaptic transmission and induction of high-frequency bursts of spikes during cyclical periods of depolarization and repolarization. Although it is not known whether the origins of this bursting are intrinsic or extrinsic to GIs, initiation of the cyclical activity does not involve cercal afferent fibers. One functional consequence of the paraoxon-induced activity was the conduction failure of GI spikes just anterior to A6. The predictable initiation and failure of spikes at various loci during

paraoxon intoxication suggest that this compound may be a useful pharmacological tool for studying excitability and spike initiation in the GIs.

REFERENCES

Burt, P. E., Gregory, G. E., and F. M. Molloy. 1966. A histochemical and electrophysiological study of the action of diazoxon on cholinesterase activity and nerve conduction in ganglia of the cockroach Periplaneta americana L. Ann. Appl. Biol. 58:341-354.

Callec, J. C. 1974. Synaptic transmission in the central nervous system of insects. Pages 119-185 in J. E. Treherne, ed. Insect Neurobiology. Plenum Press, New York.

Camhi, J. M. 1976. Non-rhythmic sensory inputs: influence on locomotory outputs in arthropods. Pages 561-586 in R. M. Herman, S. Grillner, P. S. G. Stein, and D. G. Stuart, eds. Neural Control of Locomotion. Plenum Press, New York.

Carlson, C. G., and W.-D. Dettbarn. 1983. A comparison of the effects of acute and chronic cholinesterase inactivation on spontaneous transmitter release. Brain Research 264:285-292.

Colhoun, E. H. 1960. Acetylcholine in Periplaneta americana L. IV. The significance of esterase inhibition in intoxication, acetylcholine levels, and nervous conduction. Can. J. Biochem. Physiol. 38:1363-1960.

Daley, D. L., Vardi, N. G., Appignani, B., and J. M. Camhi. 1981. Morphology of the giant interneurons and cercal nerve projections of the American cockroach. J. Comp. Neurol. 196:41-52.

Eccles, J. C., and W. V. McFarlane. 1949. Actions of anti-cholinesterases on end plate potential of frog muscle. J. Neurophysiol. 12:59-80.

Farley, R. D., and N. S. Milburn. 1969. Structure and function of the giant fibre system in the cockroach, Periplaneta americana. J. Insect Physiol. 15:457-476.

Harris, C. L., and T. Smyth. 1971. Structural details of cockroach giant axons revealed by injected dye. Comp. Biochem. Physiol. 40A:295-303.

Harrow, I. D., Hue, B., Pelhate, M., and D. B. Sattelle. 1980. Cockroach giant interneurons stained by cobalt-backfilling of dissected axons. J. Exp. Biol. 84:341-343.

Heppner, T. J., Coats, J. R., and C. D. Drewes. in press. Neurotoxic actions of isofenphos and three isofenphos metabolites in the American cockroach, Periplaneta americana. Pestic. Biochem. Physiol.

Laskowski, M. B., and W.-D. Dettbarn. 1979. An electrophysiological analysis of the effects of paraoxon at the neuromuscular junction. J. Pharmacol. Exp. Ther. 210:269-274.

Narahashi, T. 1971. Effects of insecticides on excitable tissues. Pages 1-93 in J. W. L. Beament, J. E. Treherne, and V. B. Wigglesworth, eds. Advances in Insect Physiology. Academic Press, New York.

Narahashi, T., and T. Yamasaki. 1960. Nervous and cholinesterase activities in the cockroach as affected by demeton and methyl demeton. Japan. J. Appl. Entomol. Zool. 4:64-69.

Parnas, I., Spira, M. E., Werman, R., and F. Bergmann. 1969. Non-homogenous conduction in giant axons of the nerve cord of Periplaneta americana. J. Exp. Biol. 50:635-649.

Ritzmann, R. E. 1984. The cockroach escape response. Pages 93-131 in R. C. Eaton, ed. Neural Mechanisms of Startle Behavior. Plenum Press, New York.

Roeder, K. D., Kennedy, N. K., and E. A. Samson. 1947. Synaptic conduction to giant fibers of the cockroach and the action of anticholinesterases. J. Neurophysiol. 10:1-10.

Sattelle, D. B. 1978. Insect central nervous system as a site of action of neurotoxicants. Pages 7-26 in D. L. Shankland, R. M. Hollingworth, and T. Smith Jr., eds. Pesticide and Venom Neurotoxicity. Plenum Press, New York.

Sattelle, D. B. 1980. Acetylcholine receptors of insects. Pages 215-315 in M. J. Berridge, J. E. Treherne and V. B. Wigglesworth, eds. Advances in Insect Physiology. Academic Press, New York.

Sattelle, D. B., and I. D. Harrow. 1983. α-Bungarotoxin blocks excitatory synaptic transmission between cercal sensory neurones and giant interneurone 2 of the cockroach Periplaneta americana. J. Exp. Biol. 107:473-489.

Sattelle, D. B., McClay, A. S., Dowson, R. J., and J. J. Callec. 1976. The pharmacology of an insect ganglion: Actions of carbamylcholine and acetylcholine. J. Exp. Biol. 64:13-23.

Shankland, D. L., Rose, J. A., and C. Donniger. 1971. The cholinergic nature of the cercal nerve-giant fiber synapse in the sixth abdominal ganglion of the American cockroach, Periplaneta americana (L.). J. Neurobiol. 2:247-262.

Spira, M. E., Parnas, I., and F. Bergmann. 1969b. Histological and electrophysiological studies on the giant axons of the cockroach Periplaneta americana. J. Exp. Biol. 50:629-634.

Spira, M. E. , Yarom, Y., and I. Parnas. 1976. Modulation of spike frequency by regions of special axonal geometry and by synaptic inputs. J. Neurophysiol. 39:882-899.

Twarog, B. M., and K. D. Roeder. 1957. Pharmacological observations on the desheathed last abdominal ganglion of the cockroach. Ann. Entomol. Soc. Amer. 50:221-237.

Yamasaki, T., and T. Narahashi. 1958. Synaptic transmission in the cockroach. Nature 182:1805-1806.

Yamasaki, T., and T. Narahashi. 1960. Synaptic transmission in the last abdominal ganglion of the cockroach. J. Insect Physiol. 4:1-13.

GENERAL SUMMARY

Part I. In this study it was shown that isofenphos was much less potent than some of its own oxon metabolites in inducing electrical activity in the ventral nerve cord and inhibiting ventral nerve cord cholinesterase. The metabolite des-N-isopropyl oxon (DNIO) was an expecially potent inhibitor of cholinesterase *in vivo*, suggesting that the neurotoxic effects of isofenphos could be attributed to the bioactivation and subsequent actions of the isofenphos metabolites on cholinesterase.

Part II. The results from this study provide new insight into the cellular mechanism of action of paraoxon on insect neurons. Paraoxon caused: 1) synaptic block at the cercal afferent-to-giant interneuron synapse 2) cyclical depolarizations and repolarizations with accompanying high-frequency spiking 3) failure of spike propagation during high frequency repetitive firing. The latter two effects appear to be unrelated to the cercal sensory inputs onto GIs, but further studies will be required to determine if the sites of paraoxon action are intrinsic or extrinsic to the giant interneurons.

TAPE CODE #_____

RATER'S NAME_____

EXPERIENCED

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FRIENDLY

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HONEST

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SOCIABLE

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SINCERE

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SKILLFUL

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CONFRONTIVE

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